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DNA Adduct Formation by 7,12-Dimethylbenz(a)anthracene and its Noncarcinogenic 2-Fluoro Analogue in Female Sprague-Dawley Rats

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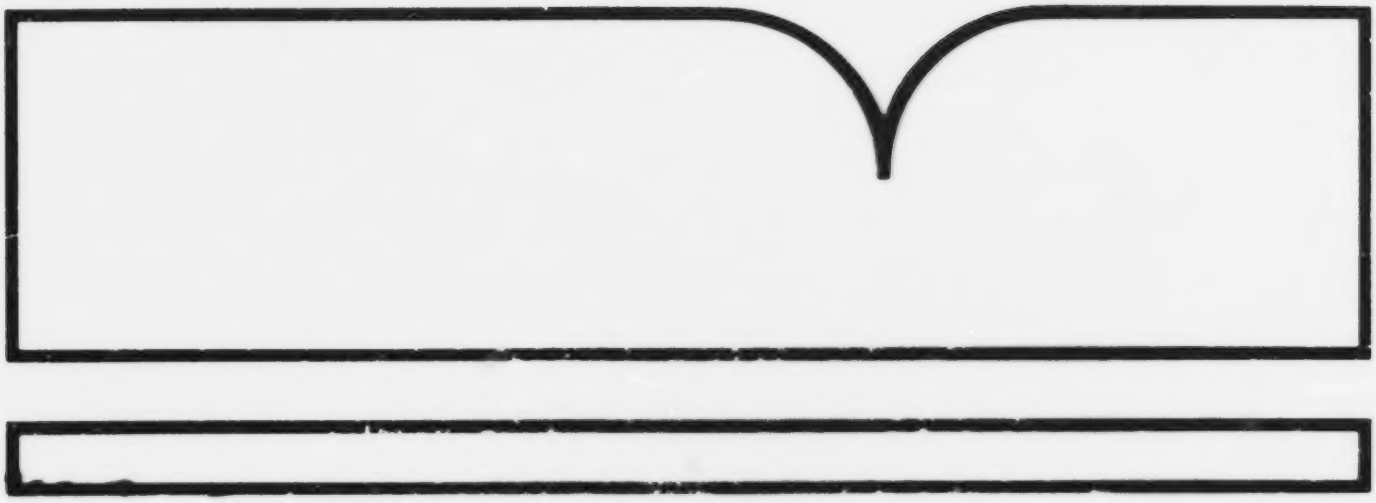

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16. ABSTRACT

The potent polycyclic aromatic hydrocarbon 7,2 dimethylbenz[a]anthracene (DMBA) bound to the DNA of numerous organs of the female outbred Sprague-Dawley rat after iv administration under a regimen known to produce a high yield of mammary adenocarcinomas. The maximum DNA binding levels observed following iv administration of 5 mg (20 μmol) DMBA range from approximately 12 μmol hydrocarbon/mol deoxyribonucleic for the liver to approximately 5 μmol hydrocarbon/mol deoxyribonucleotide for the mammary gland, the target tissue. Administered under identical conditions, the noncarcinogenic analogue 2-fluoro-7,12-dimethylbenz[a]anthracene (2F-DMBA) bound to the DNA at levels of about 5-10% that of DMBA (i.e., 0.3-1.6 μmol/mol deoxyribonucleotide). Chromatographic analysis of the hydrocarbon-deoxyribonucleoside adducts produced showed that for DMBA at least two major types of identifiable adducts were observed in all tissues examined, the major one being that resulting from the reaction of a DMBA bay-region diol-epoxide. The other adduct type resulted from the binding of an analogous diol-epoxide of a DMBA metabolite, 7-hydroxymethyl-12-methylbenz[a]anthracene. ✓ Adducts ✓from ✓2F-DMBA were observed on high-pressure liquid chromatography but were in quantities insufficient for characterization. The finding of higher levels of chromatography identical DMBA-DNA adducts in the nontarget (liver) tissue than in the target tissue indicated that adduct formation per se was not a sufficient stimulus for the cancer induction. However, the failure of the structurally similar 2F-DMBA, which produced only very low levels of DNA adducts, to induce mammary cancer implies that certain levels of carcinogen-DNA adducts may be necessary for carcinogenesis.

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DNA Adduct Formation by 7,12-Dimethylbenz[*a*]anthracene and Its Noncarcinogenic 2-Fluoro Analogue in Female Sprague-Dawley Rats[1,2]

F. B. Daniel[3] *and* **N. J. Joyce**[3,4]

ABSTRACT—The potent polycyclic aromatic hydrocarbon 7,12 dimethylbenz[*a*]anthracene (DMBA) bound to the DNA of numerous organs of the female outbred Sprague-Dawley rat after iv administration under a regimen known to produce a high yield of mammary adenocarcinomas. The maximum DNA binding levels observed following iv administration of 5 mg (20 μmol) DMBA range from approximately 12 μmol hydrocarbon/mol deoxyribonucleic for the liver to approximately 5 μmol hydrocarbon/mol deoxyribonucleotide for the mammary gland, the target tissue. Administered under identical conditions, the noncarcinogenic analogue 2-fluoro-7,12-dimethylbenz[*a*]anthracene (2F-DMBA) bound to the DNA at levels of about 5–10% that of DMBA (i.e., 0.3–1.6 μmol/mol deoxyribonucleotide). Chromatographic analysis of the hydrocarbon–deoxyribonucleoside adducts produced showed that for DMBA at least two major types of identifiable adducts were observed in all tissues examined, the major one being that resulting from the reaction of a DMBA bay-region diol-epoxide. The other adduct type resulted from the binding of an analogous diol-epoxide of a DMBA metabolite, 7-hydroxymethyl-12-methylbenz[*a*]anthracene. Adducts from 2F-DMBA were observed on high-pressure liquid chromatography but were in quantities insufficient for characterization. The finding of higher levels of chromatographically identical DMBA–DNA adducts in the nontarget (liver) tissue than in the target tissue indicated that adduct formation per se was not a sufficient stimulus for the cancer induction. However, the failure of the structurally similar 2F-DMBA, which produced only very low levels of DNA adducts, to induce mammary cancer implies that certain levels of carcinogen–DNA adducts may be necessary for carcinogenesis.—JNCI 1983; 70:111–118.

DMBA (text-fig. 1: **1**) is the most potent PAH carcinogen toward mouse skin yet studied. As little as 1 nmol of this chemical (followed by a suitable promoter) will produce tumors in Sencar mice (*1*). Likewise, DMBA is highly carcinogenic to the mammary gland of adolescent female Sprague-Dawley rats. The iv injection (*2–4*) or ip administration (*5*) of 20 μmol (5 mg) DMBA into 50-day-old Sprague-Dawley female rats will initiate mammary adenocarcinoma in virtually all of the treated animals. In contrast, DMBA under these conditions is not oncogenic toward the liver of these animals.

DMBA has been shown to bind to DNA subsequent to the formation of a diol-epoxide in the angular (A-ring) (*6–13*) as predicted by the "bay-region" theory (*14*) of PAH carcinogenesis. An earlier study from this laboratory demonstrated that such A-ring diol-epoxide (text-fig. 1: **4, 5**) of both DMBA and one of its monohydroxymethyl metabolites (7OHM-12MBA) gives rise to at least some of the hydrocarbon–deoxyribonucleoside adducts obtained from female Sprague-Dawley rats treated ip with DMBA (*15, 16*).

Substitution of fluorine for hydrogen into various positions of DMBA (text-fig. 1) greatly diminishes the hydrocarbon's carcinogenic potency in several test systems (*17, 18*). For example, 2F-DMBA is essentially noncarcinogenic and nonmutagenic in the commonly used test systems (*17, 18*). Accordingly, this laboratory reported earlier that substitution of fluorine for hydrogen at C-2 and of fluorine for hydrogen at C-5 in DMBA blocks metabolism at those positions and greatly diminishes the DNA binding potential in Syrian hamster embryo cells in vitro (*19, 20*). In contrast, substitution of fluorine at C-11, i.e., 11-fluoro-DMBA, only slightly diminishes the hydrocarbon's carcinogenicity (and/or mutagenicity) (*17, 18*) and does not reduce its DNA binding potential in Syrian hamster embryo cells (*19*). These observations, as well as those in this paper, are consistent with the bay-region theory proposed by Lehr et al. (*14*), which for DMBA involves formation of a diol-epoxide in the A-ring (text-fig. 1: **4**).

This paper is an evaluation of the DNA adduct formation of DMBA and its noncarcinogenic analogue 2F-DMBA in liver (nontarget organ) and mammary gland (target organ) DNA of female Sprague-Dawley rats. This study expands (via adduct analysis) and confirms an earlier preliminary report from our laboratory on DMBA and 2F-DMBA adduct formation in vivo (*21*).

MATERIALS AND METHODS

Hydrocarbons.—Generally, tritiated DMBA was purchased from Amersham Corp., Arlington Heights, Ill., and purified with unlabeled compound (Eastman Organic Chemicals, Rochester, N.Y.) as previously described (*21*). All preparations were at least 95% radiochemically pure by HPLC and were assayed on the day of usage. The 2F-DMBA was prepared by procedures in (*22*) and generally tritiated by Amersham Corp. This compound was purified in the same manner as DMBA.

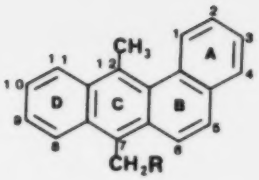
Abbreviations used: CCM=Chromatographic Control Module; DHD=dihydrodiol; diol-epoxide=1,2,3,4-tetrahydro-3,4-dihydroxy-1,2-oxide; DMBA=7,12-dimethylbenz[*a*]anthracene; 2F-DMBA=2-fluoro-DMBA; FU=relative fluorescence units; DMSO=dimethyl sulfoxide; HPLC=high-pressure liquid chromatography; LDC=Laboratory Data Control; 7OHM-12MBA=7-hydroxymethyl-12-methylbenz[*a*]anthracene; PAH=polynuclear aromatic hydrocarbon; PBS=phosphate-buffered saline; PNP=4-(*p*-nitrobenzyl)-pyridine.

[1] Received February 17, 1982; accepted August 13, 1982.

[2] Guidelines for the care and use of laboratory animals were followed as set forth by the National Research Council.

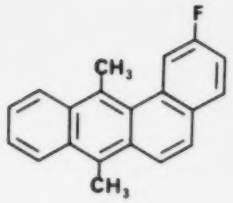
[3] Health Effects Research Laboratory, U.S. Environmental Protection Agency, 26 West St. Clair St., Cincinnati, Ohio 45268.

[4] We acknowledge the helpful discussion of our collaborators Dr. S. M. D'Ambrosio and Dr. C. T. Oravec, The Ohio State University.



1 R=H

2 R=OH



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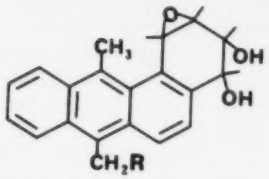


4 R=H

5 R=OH

TEXT-FIGURE 1.—Line structures of chemicals referred to in this text. 1) Ring numbering and lettering squence for DMBA. Bay-region refers to that area between C-1 and C-12; 2) 7OHM-12MBA; 3) 2F-DMBA; 4, 5) bay-region diol-epoxides of DMBA and 7OHM-12MBA, respectively.

Preparation of hydrocarbon–DNA adducts.—Forty-day-old female Sprague-Dawley outbred rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. These animals received injections at 50 days of age via the tail vein with 5.12 mg DMBA (20 μmol)/animal [sp act, 51.2 mCi/mmol dissolved in 0.5 ml DMSO–PBS (95:5, vol/vol) (*23*)]. In the case of 2F-DMBA the 50-day-old animals were given injections of 5.96 mg (20 μmol)/animal (sp act, 57.3 mCi/mmol in the same solvent as for DMBA). This regimen represents a dose of about 130 μmol hydrocarbon/kg body weight.

All animals were housed in a temperature-controlled (22–23°C) room with artificial lighting (12-hr light) and with food and water ad libitum. Mammary glands were collected at designated time points following anesthesia with a 0.25-ml im injection of a 1:10 mixture of acepromazine malate (10 mg/ml)–ketamine HCl (100 mg/ml). Other organs were removed immediately after the mammary tissue was excised, washed in PBS, weighed, immediately frozen in liquid nitrogen, and stored at −70°C until DNA isolation. Rats were killed and tissues were processed under yellow (Westinghouse FG040) lamps.

DNA isolation.—Frozen mammary tissue was cut into small (3 mm^3) pieces and treated with collagenase III (Worthington Biochemical Corp., Freehold, N.J.) (9 mg/g tissue) at 37°C for 60 minutes in PBS. The digest was then centrifuged at 1,000 rpm for 15 minutes, and the upper layer of lipids and fat cells were removed by aspiration. The cell pellet was washed with PBS, and an aliquot was examined under a Zeiss UPL phase-contrast microscope (× 100). These preparations were predominantly mammary ductal material and were used as the source for mammary DNA. An equal volume of 1.5% sodium dodecyl sulfate was added to the cell pellet, and autodigested pronase B (Calbiochem-Behring Corp., La Jolla, Calif.) was added at 100 μg/ml; the pellet was then homogenized at 100 rpm for 5 seconds with the use of a Tissumizer equipped with a microprobe. The homogenate was incubated for 60 minutes at 37°C, then made 6% in *p*-aminosalicylic acid and 1% in NaCl, and extracted with one volume of chloroform–isopentyl alcohol (24:1, vol/vol). The resulting aqueous was extracted for 30 minutes with one volume of Kirby's phenol reagent (*24*), and after centrifugation (10,000×*g*, 5°C, 30 min) the nucleic acids were precipitated from the upper aqueous layer by addition of 1.5 volumes of 2-ethoxyethanol. The nucleic acid precipitate was washed twice with ethanol and twice with ethyl ether, redissolved in a minimum volume of 0.1 *M* Tris–0.01 *M* EDTA buffer, and treated with 50 μg RNase A/ml (heat treated) and 50 μg α-amylase/ml for 30 minutes at 37°C, followed by pronase B (50 μg/ml) for 30 minutes at 37°C. The solution was made 1% with respect to NaCl, and the DNA was precipitated by addition of 1.5 volumes of 2-ethoxyethanol. The pellet was washed twice with ethanol and ethyl ether, redissolved in 0.1 *M* Tris (pH 7.7), and analyzed by reaction of a small aliquot with 3,5-diamino-benzoic acid (*25*). These DNA preparations contained less than 1% by weight protein (Bio-Rad protein assay) and typically have 260 nm:230 nm and 260 nm:280 nm absorbance ratios of 2.3 and 1.8, respectively. The binding level was calculated by treatment of an aliquot of the DNA with DNase I followed by the addition of PCS (Amersham Corp.) and by liquid scintillation analysis. The DNA of the other organs was isolated in a similar manner except that the collagenase treatment was omitted (*15*).

DNA hydrolysis.—The purified DNA was hydrolyzed by the addition of 0.1 *M* Tris buffer with 0.01 *M* $MgCl_2$, 0.005 *M* $CaCl_2$, 0.001 *M* $ZnCl_2$ (pH 8.3 at 25°C), small amounts of DNase I, and snake venom phosphodiesterase. After incubation at 37°C for 4–6 hours, bacterial alkaline phosphatase (all enzymes from Worthington Biochemical Corp.) was added and the solution was incubated at 37°C for 14–16 hours. Samples were checked by thin-layer chromatography to ensure completeness of hydrolysis to the deoxyribonucleoside level (*15*).

Chromatography.—The Sephadex LH-20 system, previously described in detail (*19, 26*), consisted of a 0.9×33-cm column (5.7 g gel) eluted with 0–100% methanol in water gradient. A small amount of PNP was added to each hydrolysate immediately before chromatography to serve as a UV reference marker.

Separation of the hydrocarbon–deoxyribonucleoside adducts from the unmodified deoxyribonucleosides in the hydrolysates was achieved by extraction of the hydrolysate with an equal volume of water-saturated *n*-butanol. After removal of the *n*-butanol under an N_2 stream, the hydrocarbon–deoxyribonucleoside adducts were redissolved in water–methanol for HPLC analysis. The HPLC gradient system consisted of a DuPont-Zorbax ODS column (4.6×250 mm) with a 100-minute linear and 0–100% methanol in water gradient eluted at 1 ml/minute by an LDC chro-

matographic system controlled by an LDC CCM (Laboratory Data Control, Riviera Beach, Fla.). The effluent was monitored by an LDC spectromonitor-III UV detector set at 290 nm and an LDC fluoromonitor-III with excitation at 254 nm and a 470-nm emission filter. The spectral detector peak areas were integrated by the LDC CCM. Fractions of 1 ml were gathered by an ISCO model 1,800-fraction collector fitted with a model 590 flow interrupter. The 100 1-ml fractions were enumerated in an LKB Laboratories model 1216 scintillation counter equipped with a DPM-PLOT package, after mixing occurred with 5 ml PCS (Amersham Corp.).

RESULTS

Administration of 5 mg DMBA iv to 50-day-old Sprague-Dawley female rats (165±12.1 gm) resulted in rapid activation and DNA binding of the hydrocarbon to all of the tissues that have been studied (heart, lung, liver, kidney, spleen, and mammary) (*21*). Maximum DNA binding for this dose administered iv occurred between 24 and 48 hours in all tissues studied. Table 1 summarizes the 24-hour liver and mammary DNA binding levels obtained in three experiments performed over a 2-year period. Due to large interanimal variation, low DNA binding, and small amounts of mammary DNA obtainable, it is recommended that at least 4 animals per time dose point be employed for mammary DNA binding studies.

Table 2 compares the hydrocarbon–deoxyribonucleoside adduct levels for DMBA and 2F-DMBA in the liver and mammary gland for one experiment (expt III) as a function of time after iv administration of the hydrocarbon. Consistent with an earlier study with Syrian hamster embryo cells, DMBA bound to DNA at a much higher level than did 2F-DMBA (*19*). In addition, there was little evidence of removal of the adducts over the time span of the experiments (168 hr).

Since at least some of the tritium could be expected to be labilized during the metabolism of a generally tritiated PAH, it would be preferable for chromatographic analysis of the DNA hydrolysates to be included when measurement of binding levels is attempted. Text-figure 2 shows a Sephadex LH-20 chromatogram from a liver DNA hydrolysate and is typical of those obtained in our laboratory for all rat tissues. This profile was included for the purpose of giving continuity between this study and numerous previous studies employing this method of chromatography. The polar radioactivity (LH1, LH2 fractions 10–22; text-fig. 2) eluting in the high-water portion of the gradient has historically been observed by other investigators (*10, 27*). The chemical nature of this early polar material has not been characterized. As a percentage of the total radioactivity the early polar material varies from experiment to experiment, ranging from 30 to 50% of the total radioactivity isolated with the DNA.

In addition to the polar radioactivity, we invariably observed at least two other radioactive products eluting in the midrange of these Sephadex LH-20 gradient chromatograms, LH3 (fractions 48–53) and LH4 (fractions 60–67). Occasionally, a predominant shoulder, LH5, was observed on the high-fraction side of LH4 (text-fig. 2). Previously, we have presented chromatographic and spectrofluorometric evidence showing that LH3 is a deoxyribonucleoside adduct of 7OHM-12MBA, one of the monohydroxymethyl metabolites of DMBA (*15, 16*). Likewise, LH4 has been shown to be an A-ring adduct to DMBA (*15, 16*), and the trailing shoulder LH5 has not been characterized.

Table 3 summarizes the relative amounts of LH3 and LH4 observed on Sephadex LH-20 chromatograms from several tissues. An apparent difference in the ratio of the amounts of LH3 to LH4 was observed between various tissues, e.g., 0.34–0.57 liver versus 0.1–0.2 for the mammary; however, these data were considered tentative since the very small amount of DNA available from the mammary glands (≈100 μg/animal) made analysis of these chromatograms relatively more difficult.

To more fully resolve the hydrocarbon–deoxyribonucleo-

TABLE 1.—*Twenty-four-hour liver and mammary DNA binding of DMBA*[a]

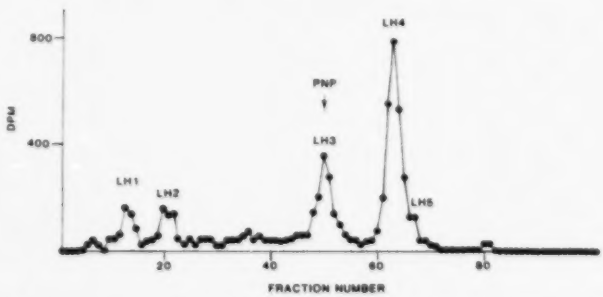
Expt No.	Binding level	
	Liver	Mammary gland
I	10.6	6.1
II	9.6±1.8	4.0±0.1
III	12.4±3.8	5.3±1.0

[a] Binding levels expressed as μmol DMBA/mol deoxyribonucleoside, on the basis of total radioactivity isolated with the DNA. Expt I, 2–3 animal pools/point; expt II, 2 animals/point ± SD; expt III, 4 animals/point ± SD. For all experiments, animals were dosed iv with 20 μmol DMBA.

TABLE 2.—*Binding of DMBA and 2F-DMBA to liver and mammary gland DNA as a function of time post administration*[a]

Hour	Liver		Mammary gland	
	DMBA	2F-DMBA	DMBA	2F-DMBA
4	3.1±1.9	0.7±0.4	1.5±1.1	0.4±0.2
24	12.4±3.8	1.6±0.4	5.3±1.0	0.3±0.1
48	11.6±5.9	0.8±0.3	5.0±0.6	0.3±0.1
168	9.3±4.4	0.6±0.3	4.8±0.5	0.3±0.1

[a] Data from expt III, 4 animals/point; data are μmol hydrocarbon/mol deoxyribonucleotide ± SD and are based on total radioactivity associated with the purified DNA.



TEXT-FIGURE 2.—Sephadex LH-20 chromatogram of a liver DNA hydrolysate from a female Sprague-Dawley rat treated 168 hr previously with 5 mg [^{3}H]DMBA. *Arrow* leads to the elution point of PNP.

TABLE 3.—*Sephadex LH-20 DMBA-deoxyribonucleotide binding data*

Tissue	Expt No.	Hour	Total binding[a]	LH3:LH4[b]
Kidney[c]	I	6	5.9	0.26
		24	20.9	0.32
		168	14.5	0.35
Lung	I	6	8.5	0.30
		24	7.5	0.30
Mammary[d]	I	6	2.2	0.22
		24	6.1	0.10
		168	4.1	0.07
Liver	I	6	8.8	0.34
		24	10.6	0.55
		168	4.9	0.51
	II	4	3.9	0.56
			8.2	0.41
		24	10.9	0.56
			8.3	0.54
	IV[e]	12	7.6	0.47
		36	11.9	0.50
		60	9.4	0.48
		192	10.4	0.57

[a] μmol DMBA/mol deoxyribonucleotide on the basis of total radioactivity isolated with DNA.

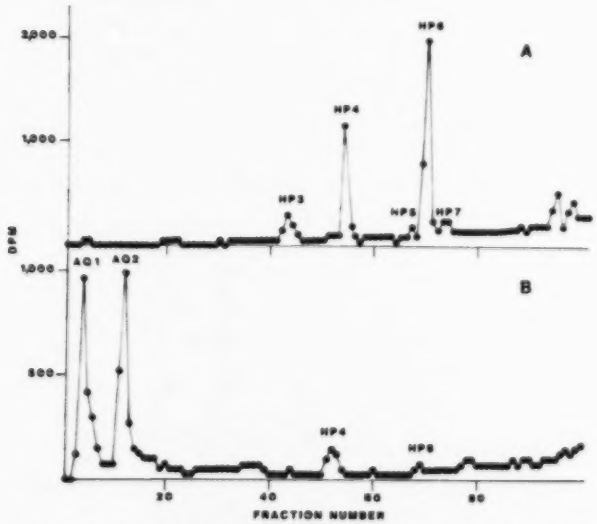
[b] On the basis of dpm under peaks from LH-20 chromatograms.

[c] Kidney DNA binding was higher than that found in subsequent experiments; the usual range is 3–7 μmol DMBA/mol deoxyribonucleotide; data are included since chromatography was performed on these samples.

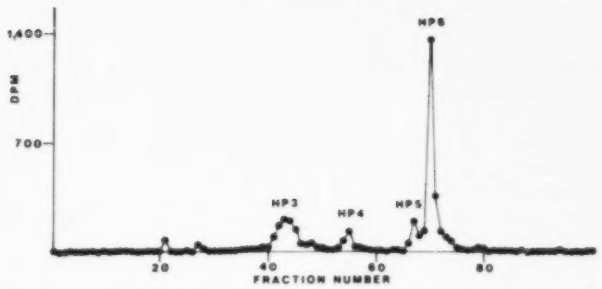
[d] Value is best estimate from Sephadex LH-20 chromatogram. *See* table 4.

[e] Experiment IV, 4 animals/point.

side adducts, HPLC was employed. Text-figure 3 depicts the profile of a portion of a 168-hour liver hydrolysate analyzed by HPLC. The upper trace (A) is the organic phase of the hydrolysate following a single *n*-butanol extraction, while the lower chromatogram (B) represents analysis



TEXT-FIGURE 3.—A) An HPLC chromatogram of the *n*-butanol extract of a liver DNA hydrolysate from rats treated with 5 mg [^{3}H]DMBA 168 hr previously; B) an HPLC of a portion of the aqueous phase from the extracted hydrolysate.



TEXT-FIGURE 4.—An HPLC chromatogram of the *n*-butanol extract of a mammary DNA hydrolysate from female Sprague-Dawley rats treated with 5 mg [^{3}H]DMBA 168 hr before they were killed.

of a portion of the aqueous layer from the extraction on the same gradient system. Text-figure 4 depicts the chromatogram of the organic phase of a 168-hour mammary DNA hydrolysate prepared from a pool of 10 animals (*see* table 4). The aqueous phase was too dilute for chromatography. The HPLC on Zorbax-ODS provided somewhat greater resolution of the adducts. The polar materials LH1 and LH2 appeared as AQ1 and AQ2 on the HPLC trace of the extracted aqueous. The chemical nature of HP3 was unknown, whereas HP4 corresponded to LH3. The major peaks LH4 and shoulder LH5 (from Sephadex LH-20) were resolved when injected on HPLC into HP5, HP6, and HP7 (text-figs. 3, 4). The adducts HP4 through HP7 were all detected by the HPLC fluorescence detector (data not shown). A portion of HP4 (text-fig. 3) remained in the aqueous layer following a single *n*-butanol extraction; thus care must be exercised when an attempt is made to evaluate the level of this adduct via HPLC. Two successive *n*-butanol extractions of the DNA hydrolysate completely removed HP4 from the aqueous phase (data not shown).

Use of the HPLC system allowed reevaluation of the question of selective removal of the various DMBA–deoxyribonucleoside adducts. A study of the HPLC profiles of liver DNA hydrolysates from animals killed at various times after DMBA administration showed only quantitative differences (table 4). No evidence of selective removal of various DNA adducts was found in the liver, with time. Unfortunately, there was not sufficient DNA for an analysis of each

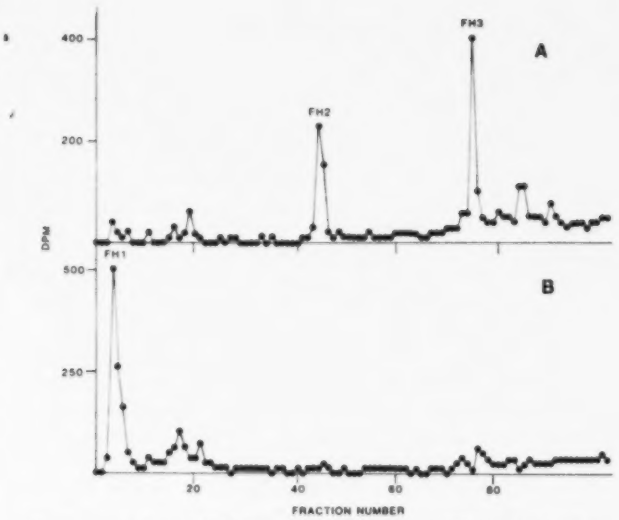
TABLE 4.—*Analysis by HPLC of DMBA–deoxyribonucleoside adducts*[a]

Tissue	Hour	HP4[b]	HP6[b]	HP4:HP6
Liver	4	0.3	0.7	0.43
	24	0.6	1.8	0.33
	48	0.8	1.9	0.42
	168	0.6	2.2	0.27
Mammary	24, 48, 168	0.2	0.95	0.21
	168[c]	0.12	0.92	0.13

[a] Represents that portion of radioactivity extractable in the *n*-butanol phase, generally 50–70% of the total activity isolated with the DNA. Adducts HP4 and HP6 extracted from the DNA hydrolysate with the use of two *n*-butanol extractions.

[b] μmol hydrocarbon/mol deoxyribonucleotide; data are from expt III.

[c] Expt V conducted on 10 female Sprague-Dawley rats 50 days of age. Mean binding level at 168 hr was 5.11 μmol DMBA/mol deoxyribonucleotide.



TEXT-FIGURE 5.—An HPLC analysis of the rat liver DNA hydrolysate from animals treated 168 hr previously with [^{3}H]2F-DMBA. A) Chromatogram of the *n*-butanol phase of an extracted hydrolysate. B) Chromatogram of aqueous phase of the extracted hydrolysate.

time point for the mammary gland; however, analysis was made of the 24-, 48-, and 168-hour pooled mammary DNA samples (table 4). To obtain more mammary DNA, an additional experiment (expt V, table 4) was performed in which the mammary glands from 10 rats all killed at 168 hours were analyzed. This experiment gave results in excellent agreement with that of the pooled sample (table 4; text-fig. 4). In concordance with the Sephadex LH-20 studies, the liver DNA contained relatively higher levels of HP4 (LH3). We have previously shown that HP4 co-chromatographs with the 7OHM-12MBA–deoxyribonucleoside adducts obtained from the liver of female Sprague-Dawley rats dosed with [3H]7OHM-12MBA (*15, 16*).

The binding levels of the [^{3}H]2F-DMBA to both liver and mammary DNA were on the order of 5–10% of that for DMBA (table 2). These levels compared favorably with those obtained in earlier comparative binding studies performed in Syrian hamster embryo cells (*19*) and in the Sprague-Dawley rat (*21*). When liver DNA hydrolysates from animals previously treated with [^{3}H]2F-DMBA were subjected to HPLC the pattern shown in text-figure 5 was obtained. Two radioactive products were routinely observed at fractions 42–46 (FH2) and 73–77 (FH3). The chromatographic trace shown in text-figure 5B was analogous to that in text-figure 3B and represented the aqueous phase of the hydrolysate after *n*-butanol extraction. The supposed adducts FH2 and FH3 exhibited very little fluorescence intensity under the detector settings used to monitor the DMBA–deoxyribonucleoside adducts (HPLC). The low levels of 2F-DMBA binding obtainable have precluded the possibility of chromatographic analysis of the mammary DNA samples.

DISCUSSION

DMBA is a potent carcinogen for the mammary gland of the developing female Sprague-Dawley rat (*2*). As little as 5 mg (≈20 μmol) given iv (*2, 4*) or ip (*5*) will induce a high yield of mammary adenocarcinoma in 5–6 weeks. Nearly identical results are obtained with a single intragastric dose of 20 mg (≈78 μmol) (*28, 29*). The tumor incidence for a 5-mg oral dose in these animals fed a commercial laboratory diet is lower with 20–30% of the animals developing mammary adenocarcinoma in 17 weeks [(*28, 30*); Ip C: Personal communication]. However, for animals fed a synthetic diet containing a fat content equivalent to that of laboratory chow (5%), the tumor incidence following a 5-mg oral dose of DMBA is somewhat higher [(*30*); Ip C: Personal communication]. Ip et al. (*31, 32*) have clearly established that dietary fat is a promoter for DMBA-induced mammary cancer in the Sprague-Dawley rats.

The most sensitive age with respect to polycyclic hydrocarbon-induced mammary tumorigenesis for the female Sprague-Dawley rat is 50–67 days old, with older animals being considerably less sensitive (*28*). Russo and Russo (*33*) have recently confirmed this observation and proposed that this is the period of mammary gland differentiation and development with concomitant high levels of DNA synthesis (*34, 35*). Earlier, Nagasawa et al. (*36*) also proposed that the explanation lies in the fact that the level of DNA synthesis at the time of DMBA insult was a critical factor in the susceptibility of the mammary gland to DMBA-induced cancer. Therefore, the Sprague-Dawley rat liver normally refractory to DMBA-induced carcinogenesis is rendered susceptible if dosed shortly after partial hepatectomy (*37, 38*).

DMBA has been shown to bind to cellular DNA in a number of in vitro (*8–13, 19, 26*) and in vivo systems (*15, 16, 21, 27, 37, 39–41*) following cellular metabolism. The bay-region theory of polycyclic aromatic hydrocarbon carcinogenesis predicts that DMBA will be activated to DNA binding forms via the formation of a diol-epoxide in the angular benzo ring (A-ring) leading eventually to a reactive benzyl carbonium ion that can subsequently react at nucleophilic centers on the DNA and other cellular molecules. Considerable evidence indicates that such carcinogen–DNA interactions are responsible for initiation of the carcinogenic process (*42, 43*).

Although the critical postulated metabolite of DMBA—DMBA-diol-epoxide (text-fig. 1, 4)—has never been synthetically prepared and characterized, evidence from several laboratories indicates that it is the major DNA binding form in cultured rodent embryo cells (*6, 7, 9, 11–13*), in mouse skin in vivo (*8, 10*), and in rat tissues in vivo (*15, 16, 41*). Recently, this laboratory has shown that the DNA digests from the livers of female Sprague-Dawley rats previously treated ip with DMBA contain two major types of hydrocarbon–deoxyribonucleoside adducts (LH3 and LH4), which elute in the midregion of Sephadex LH-20 chromatograms (*15, 16*). These two adducts were further purified by HPLC to HP4 and HP6, respectively. [In (*15, 16*), HP4 was designated III and HP6 as IV.] The adducts HP4 and HP6 were shown to be A-ring hydrocarbon–deoxyribonucleoside adducts of the diol-epoxides of 7OHM-12MBA and DMBA, respectively (*15, 16*). The evidence is summarized: 1) Both HP4 and HP6 have excitation and emission spectra expected for DMBA–deoxyribonucleoside adducts containing a saturated A-ring (*7, 8*); 2) HP4 obtained from the liver

of rats treated with DMBA co-chromatographs with the adduct obtained from 7OHM-12MBA-treated animals on both HPLC and Sephadex LH-20 (*15, 16*).

It is curious that the 7OHM-12MBA–deoxyribonucleoside adduct is present in such relatively large amounts in the liver DNA hydrolysates since it requires an additional metabolic step for its formation. Further, in an earlier transplacental study of DMBA–deoxyribonucleoside adduct formation in pregnant BD IX rats, Doerjer et al. (*41*) had observed only a small amount of radioactivity eluting in the region expected for 7OHM-12MBA–deoxyribonucleoside adducts on Sephadex LH-20 columns (i.e., near the PNP standard). Likewise, in cultured rodent cells in both our laboratory (*15, 19*) and others (*6, 7, 9, 11–13, 43*) and in mouse skin (*44*) 7OHM-12MBA adducts had not been observed after administration of DMBA itself. However, a very recent report has indicated that 7OHM-12MBA adducts are formed in mouse skin following administration of the parent hydrocarbon DMBA (*42*).

The chemical nature of the minor peaks observed on these HPLC and Sephadex LH-20 chromatograms remains unidentified. The small satellite peaks (HP5, HP7) on either side of the major peak HP6 are routinely detected by the HPLC fluorescence detector. In addition, for HP5 and HP7, analysis of the amount of fluorescence (HPLC detector) relative to the mass (determined by radioactivity) was similar to that for HP6 (HP6, 25.73±1.45 FU/dpm; HP5, 26.44±5.49 FU/dpm; HP7 28.52±6.05 FU/dpm). This finding would support the idea that these minor adducts are similar, with respect to the hydrocarbon moiety, to HP6. Other investigators (*7, 12, 13*) have routinely observed evidence for minor adducts on either side of the major DMBA–deoxyribonucleoside adduct.

The chromatogram of the 2F-DMBA–deoxyribonucleoside adducts (text-fig. 5A, B) is included for symmetry. Little is known about the chemical nature of these adducts; however, it is unlikely that these products represent A-ring adducts since previous studies in our laboratory have shown that rat liver microsomes do not metabolize 2F-DMBA in the A-ring under conditions where DMBA is metabolized to DMBA-3,4-DHD (*20*). An earlier study (*19*) demonstrated that the substitution of fluorine for hydrogen at a particular carbon in DMBA effectively blocks epoxide formation at that formal bond and proposed that this was related to the greater bond energy and length of the aryl carbon–fluorine bond relative to hydrogen (*19*) since both 2F-DMBA-5,6-DHD and 2F-DMBA-8,9-DHD were detected in the metabolism of 2F-DMBA by rat liver microsomes (*19, 20*) and Syrian hamster embryo cells (Oravec CT, Daniel FB: Submitted for publication). Further, the observation that the 2F-DMBA–deoxyribonucleoside adducts do not elicit a response on the HPLC fluorescence detector (that readily detects lesser amounts of DMBA–deoxyribonucleoside A-ring adducts) confirms that these adducts are probably not A-ring in nature since previous studies have clearly shown that the substitution of fluorine for hydrogen has little effect on the fluorescence spectra of either DMBA or its metabolites [(*19, 20*); Oravec CT, Daniel FB: Submitted for publication].

In previous DMBA–DNA binding studies, Janss et al. (*39, 40*) treated female Sprague-Dawley rats with [^{3}H]-DMBA via gastric intubation and observed about a ninefold higher binding in the mammary than in the liver. This finding is in contrast to the results obtained in this intravenous study (where a 1.5- to twofold-higher binding is observed in the liver); the difference is most likely due to the route of administration. Since the Janss and Ben study (*40*) did not include chromatographic analysis, comparisons between their study and ours must be made on the basis of total radioactivity bound to the DNA isolated. Although the method used to prepare mammary ductal epithelial tissue was similar in both studies (*45*), the Janss and Ben study (*40*) employed isopyknic centrifugation for DNA isolation. The mammary DNA binding level calculated from the data of Janss and Ben (*40*) for a 10-mg oral dose of [^{3}H]DMBA to 50-day-old Sprague-Dawley rats is 8.5 μmol DMBA/mol deoxyribonucleotide at 16 hours, the time of maximum binding. This binding level compares favorably to the maximum level we observed at 24 hours for the 5-mg iv dose, 5.0±0.9 μmol DMBA/mol deoxyribonucleotide; both regimens are very effective for the induction of mammary adenocarcinomas in these animals (*2, 23, 28, 29*). In our experiments about 15–25% of the radioactivity associated with the purified DNA from the [^{3}H]DMBA-treated animals chromatographs as the major adduct HP6, and an additional 8–15% appears as HP4.

Little indication of active removal of hydrocarbon–deoxyribonucleosides is noted over the time course of these experiments. A comparison of the total binding levels (table 2) and the specific adduct levels (table 4) for the liver from experiment III shows little change from the maximum at 24 hours to the last analysis point of 168 hours. Likewise, the total adduct levels found in the mammary gland do not change appreciably over the time course of the experiments (tables 2, 3). In both the Sephadex LH-20 data (expts I, II) and the HPLC data (expt III), the relative level of the 7OHM-12MBA–deoxyribonucleoside adducts (HP4 or LH3) was less in the mammary than in the liver. Obviously, this study does not rule out the possible existence, in vivo, of a steady-state equilibrium between formation and removal of the adducts since previous investigations have indicated that the fatty tissue of the mammary gland can serve as a depot for unmetabolized DMBA (*46*). In our studies, HPLC of chloroform extracts of the mammary gland homogenates contained a considerable amount of unmetabolized DMBA even at the 168-hour time point (data not shown). Others have reported that the liver can also function as a storage site for unmetabolized DMBA (*47*).

The DNA binding of 2F-DMBA (table 3) is considerably less (6% for liver, 48 hr; 5% for mammary gland, 48 hr) than DMBA (table 2) when calculated on the basis of total radioactivity bound to DNA. This is in good agreement with our previous studies on 2F-DMBA–DNA binding in Syrian hamster embryo cells (*19*). Previously, Slaga et al. (*17*) found that 2F-DMBA was essentially inactive as a tumor initiator when administered under those conditions where DMBA was very effective. In another study, Harvey and Dunne (*18*) found 2F-DMBA inactive as a sarcomogen in Long-Evans rats under conditions where DMBA causes tumors in all of the test animals. Finally, iv administration

of 20 μmol 2F-DMBA (DMSO:PBS, 95:5, vol/vol) to 50-day-old female Sprague-Dawley rats resulted in no evidence of mammary neoplasia, whereas all of the animals treated with the same dose of DMBA had at least 1 mammary adenocarcinoma (Gould D: Unpublished observations).

Thus in this paper we demonstrate that when DMBA is administered under conditions that give rise to high yields of mammary adenocarcinomas, both DMBA–deoxyribonucleoside and 7OHM-12MBA–deoxyribonucleoside adducts are formed in both target (mammary gland) and nontarget (liver) tissues. Furthermore, in these studies in which the carcinogen was administered iv the adduct levels are higher in the liver than in the mammary gland in contrast to the findings of earlier studies (*40*) with oral administration of DMBA.

Because the structurally similar but noncarcinogenic 2F-DMBA produces only very low levels of hydrocarbon–DNA adducts relative to DMBA, it follows that at least a certain level of these adducts may be a necessary event in the carcinogenic process. However, the observation that the level of DMBA–DNA binding is higher in the nontarget (liver) tissue than in the target (mammary gland) whereas the actual hydrocarbon–deoxyribonucleoside adducts are qualitatively (HPLC) similar implies that these carcinogen–DNA adducts alone are not a sufficient stimulus for the induction of cancer. Thus other parameters (discussed above) such as the level of DNA replication at the time of carcinogen insult, the level of and/or fidelity of DNA repair processes, the presence or absence of promoting factors, and the relative sensitivity of the organ to promotional stimuli may all be factors in the total relative sensitivities of the two organs to DMBA carcinogenesis.

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